



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12Q 1/10, 1/34, 1/04	A1	(11) International Publication Number: WO 98/55644 (43) International Publication Date: 10 December 1998 (10.12.98)
(21) International Application Number: PCT/GB98/01645 (22) International Filing Date: 4 June 1998 (04.06.98) (30) Priority Data: 97303846.6 4 June 1997 (04.06.97) EP (34) Countries for which the regional or international application was filed: AT et al. (71) Applicant (for all designated States except US): FREEMAN GROUP OF HOSPITALS NHS TRUST [GB/GB]; Freeman Road, High Heaton, Newcastle-upon-Tyne NE7 7DN (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): PERRY, John, David [GB/GB]; Microbiology Dept., Freeman Hospital, Freeman Road, Newcastle-upon-Tyne, Tyne & Wear NE7 7DN (GB). FORD, Michael [GB/GB]; Microbiology Dept., Freeman Hospital, Freeman Road, Newcastle-upon-Tyne, Tyne & Wear NE7 7DN (GB). (74) Agent: GILL JENNINGS & EVERY; Broadgate House, 7 Eldon Street, London EC2M 7LH (GB).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>

(54) Title: IDENTIFICATION OF SALMONELLA**(57) Abstract**

A new culture medium for identifying the presence of *Salmonella* in enterobacteria samples, especially faeces, contains two chromogenic enzyme substrates, one of which is a substrate for α -D-galactosidase, for which *Salmonella* is positive. The other substrate is one for which *Salmonella* is negative such as β -D-galactosidase. The substrates are incorporated into an agar medium. Positive and negative results are found to be readily observable where one of the substrates is an esculetin, preferably a cyclohexenoesculetin compound in the presence of ferric ions, which produces a black colour, and the other substrate is an indoxyl compound, for instance a 5-bromo-4-chloro-3-indolyl compound which produces a green coloured enzymic reaction product.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav	TM	Turkmenistan
BF	Burkina Faso	GR	Greece		Republic of Macedonia	TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

IDENTIFICATION OF SALMONELLA

The present invention relates to processes for identifying the presence of *Salmonella* species in a sample, as well as culture media suitable for such identification processes.

5 Members of the genus *Salmonella* constitute the most important causes of food poisoning in the UK. At present, the only effective means of diagnosis involves cultural isolation of the causative organism from faeces. This however is not straightforward as specialised media and reagents are required to isolate relatively small numbers of *Salmonellae* from a massive amount of commensal flora in the guts. Selective media
10 have been developed for this purpose which rely on the visualisation of simple biochemical features such as production of hydrogen sulphide or non-fermentation of lactose.

 A useful review of five plating media for isolation of *Salmonella* species and a comparison against Hektoen enteric agar, a standard medium, is described by Dusch et
15 al in J. Clin. Microbial. (1995) 33(4), 802 to 804. All but one of the media are solid (standard agar concentration) whilst one is a semi solid reduced agar concentration medium. For the solid media, the compounds which are produced in the presence of microbial growth are selected so as to be visible to the naked eye. In order that the visualised compounds are associated with microbial colonies, those compounds must be
20 non-diffusible in the culture medium. These media typically test for two different biochemical characteristics of bacterial colonies and the results are such that positive and negative results of each of the two tests can be observed with positive or negative results of the other test. Some of the biochemical tests observe the activity of specific enzymes by the use of chromogenic substrates which are uncoloured or non-fluorescent but which
25 generate enzymic reaction products which are coloured or fluorescent and can hence be observed in the presence of the substrates. Sometimes the enzymic reaction product may react with a further component of the culture medium to generate the visible product, for instance metal ions or pH indicators, where the reaction product is an acid or base.

 It is known to include in the culture medium substrates for two different enzymes
30 which have different enzymic reaction products, each of which can be observed in the presence of the other (and of each of the substrates themselves).

One enzyme substrate which is commonly used in the identification of *Salmonella* is a substrate for β -galactosidase. *Salmonella* is generally negative for this enzyme activity, but most other members of the *Enterobacteriaceae* are positive. One β -galactosidase substrate whose enzymic reaction product is non-diffusible is 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X Gal). Other indoxyl and halogenated indoxyl compounds are useful as substrates and have reaction products which are visible and non-diffusible in agar culture media.

X-Gal is used as a substrate in Rambach medium, described *inter alia* in US-A-5194374. It is used in combination with an alkanediol, which is metabolised by *Salmonella* to form an acid reaction product which is visualised by the incorporation of a pH indicator such as neutral red.

In EP-A-0516817, a culture medium for detecting *Salmonella* comprises a chromogenic β -galactosidase substrate and, in addition, glucuronate and a pH indicator. This mixed medium is alleged to be more selective than Rambach medium since almost all *Salmonella* species tested, but few other bacterial species ferment glucuronic acid resulting in a lowering of the pH.

In WO-A-94/01952, a 5-bromo-4-chloro-3-indolyl compound which is a substrate for an esterase enzyme is used to identify *Salmonellae*, which are positive for such enzymes. The substrate is an ester of a C_{7-10} -fatty acid. It is suggested that the medium may be supplemented to eliminate non-*Salmonella* bacteria, such as using properties relating to cleavage or metabolism of β -galactosides and β -glucosides (for both of which *Salmonella* is negative).

Rambach medium and the X-gal glucuronic acid combination were found by Dusch et al to have less than optimal sensitivities. A further medium comprising xylose, lysine and Tergitol 4 has very good sensitivity and specificity. The culture medium includes the surfactant Tergitol 4 to inhibit *Proteus*, and determining hydrogen sulphide formation from sodium thiosulphate in the medium which is visualised by the incorporation of ferric ions.

It is known that *Salmonella* species produce α -galactosidase, but it is likely that that enzyme would be considered a poor marker for *Salmonella* since it is produced by many related genera, such as *Escherichia*, *Citrobacter*, *Klebsiella*, *Enterobacter* and *Shigella*.

In Acta Microbiol Hung. (1988) 35(4), 389-395 Ketyi, I. discusses the α -galactosidase activity of various species of entero-bacteria including *Salmonella*, *Shigella* and *E-coli*. He indicated that enzymic activity is a general feature of *Enterobacteriaceae*. He used melibiose, as an indicator of α -galactosidase positive strains. Melibiose is not a chromogenic compound.

In WO-A-9630543 a chromogenic β -galactosidase substrate is used in combination with a mixture of sugars including mannitol, with xylose and melibiose for identifying *Salmonella*. The sugars are cleaved to form acids and the growth medium contains a pH indicator. However the acids which change the pH are products of a series of enzymic reactions on the product of sugar metabolism.

James *et al* in App. Env. Microbiol. (1996), 62(10) 3868-170 and in J. App. Microbiol. (1996), 82, 532-536, describe a new β -galactosidase substrate for use in place of X-Gal. The substrate is a derivative of cyclohexenoescluletin, of which the aglycone released by hydrolysis by β -galactosidase forms a black-brown complex with ferric ions in the medium. The new substrate, CHE-Gal, gave good correlation with X-Gal, that is high specificity and high sensitivity for detecting β -galactosidase activity.

These cyclohexenoescluletin substrates and other escluletin derivatives are described further and claimed in WO-A-9741138 (not published at the priority date of the present invention).

One aspect of the present invention is based on the need for culture media which are very sensitive to *Salmonella* whilst being highly specific, thereby minimising subsequent confirmatory tests. These types of test often need to be carried out with the inadequately specific media of the prior art. A second aspect of the invention is based on the provision of a medium comprising two chromogenic substrates which gives readily observable results. A visual determination can be easily made of the presence and absence of enzymic reaction products of each substrate regardless of the presence or absence of the enzymic reaction product of the other substrate.

According to a first aspect of the present invention there is provided a new process in which the following steps are carried out:

1. a sample suspected of containing *Salmonella* bacteria is cultured in the presence of a nutrient,
2. the bacterial culture is contacted with each of two enzyme substrates,

3. the presence of the enzymic reaction products of each of the substrates is accessed after step 2 to determine whether or not growth of *Salmonella* species has taken place,

in which the first substrate is a substrate for an enzyme for which *Salmonella* is negative, the process being characterised in that the second substrate is a substrate for α -galactosidase and in that both substrates are chromogenic.

The present inventors believe that it is the first time that α -galactosidase has been used as a marker for *Salmonella* using an α -galactosidase specific chromogenic substrate, that is a substrate for which the enzymic product of the reaction in the presence of α -galactosidase is chromogenic without being subjected to further enzymic reactions. Thus, the inventors have recognised the utility of combining β -galactosidase and α -galactosidase as markers for detecting *Salmonella* species. The method is useful for carrying out the usual tests to identify *Salmonella*. It is not necessary for there to be any specific expectation of *Salmonella* presence in a clinical sample tested in the present invention. Thus the invention is suitable for screening to exclude *Salmonella* (giving a negative result) as well as for positive tests.

The enzyme for cleaving the said first substrate, the activity for which *Salmonella* is negative, is selected such that a positive result (cleavage) can exclude a large number of *Enterobacteriaceae*. *Salmonellae* are negative for β -glucosidase and a substrate for β -glucosidase could therefore be used. Many other enterobacteria are also negative for β -glucosidase. Best results are achieved where substrates for that enzyme are used in combination further with other enzyme substrate. Such other substrates would be selected to help distinguish *Salmonella* from such other β -glucosidase negative species. Most conveniently the first substrate is selected to be cleavable by β -galactosidase. The substrate therefore should preferably be a derivative of β -D-galactopyranoside.

Although the invention may be used in a panel of biochemical tests, each of which is carried out in an individual container, on a single bacterial colony, it is preferred that the process is used for samples containing a mixture of bacterial species which are cultured together on a body of culture medium in a single container. The culture medium is preferably a solid (gelled) medium, most conveniently based on agar. Other conventional support materials for bacterial culturing can be used.

The sample, as mentioned above, preferably contains a mixture of bacterial species. It may be a direct sample, inoculated using a suitable technique onto the culture medium. Thus it may be a sample of food, water, or bodily fluid of a patient, usually blood, urine or, most preferably faeces. Alternatively a direct sample may, prior to carrying out the process, be enriched by inoculating the direct sample into an enrichment broth and culturing the broth for a period of time, for instance 24 hours, before inoculating a portion of the bacterial culture onto the culture medium for the process of the invention. The enrichment medium is selected so as to favour the growth of *Salmonella* species over other common enterobacteria such as *E.coli* and *Proteus*. Suitable enrichment media are, for instance, tetrathionate or selenite broths.

The medium in which the first culturing step of the invention is carried out preferably includes components which favour growth of *Salmonella*. Thus the medium may contain known inhibitors of other enterobacterial growth such as brilliant green, bile salts or desoxycholate sodium salt.

Where the enzyme activity for which *Salmonella* is negative is β -galactosidase, the first step of the process of the present invention is preferably carried out in the presence of a β -galactosidase promoter of known type, for instance lactose or, preferably, isopropyl- β -D-thiogalactopyranoside.

In the process the enzyme substrates are each chromogenic. In this specification, the term chromogenic encompasses fluorogenic. The enzymic reaction products or each substrate are preferably directly visible, for instance as coloured compounds, optionally in the presence of other components such as metal ions, preferably by the naked eye in visible light. Alternatively the reaction products may be detectable spectrophotometrically, by observing absorbed radiation of any predetermined wavelength, or fluorometrically by observing fluorescence.

Alternatively the direct enzyme reaction products may be visible after further chemical, non-enzymic reaction.

It is preferred that the direct product of the enzymic cleavage is detectable without further chemical reaction, since such further reactions may be non-specific and cause false readings. The invention does not include the use of pH indicators to identity the presence of the cleavage product.

Where both substrates are in physical admixture in the same body of culture medium, the enzymic reaction products of the two substrates must be different compounds, at least one of which should be detectable in the presence of the other and in the presence of both the substrates themselves. The other reaction product might be masked by the first, or be visible in its presence. Thus any combination of positive and negative reaction can be observed in the reaction medium.

Although the step of contacting the substrates with the cultured bacteria may take place after culturing has been carried out for a period of time, and in a step in which no further bacterial growth or metabolism takes place, preferably culturing takes place in the presence of the enzyme substrates. Thus the substrates are incorporated into the culture medium at the beginning of the culturing step 1 of the process. The substrates should, consequently, be non-toxic for bacteria, or at least for *Salmonella*, allowing growth of, especially, *Salmonella*, to take place.

The present inventors have discovered that a particularly useful combination of enzymic substrates comprises a substrate which generates an enzymic reaction product which is an indoxyl compound, including a halogen substituted compound, and a second substrate which is an esculetin, especially a 3,4-cyclohexenoesculetin compound. Where the latter substrate is used, during or after contacting of the cultured bacteria with the substrate ferric ions should be contacted with the medium. This leads to generation of a brown black colour with the enzymic reaction product of such a substrate.

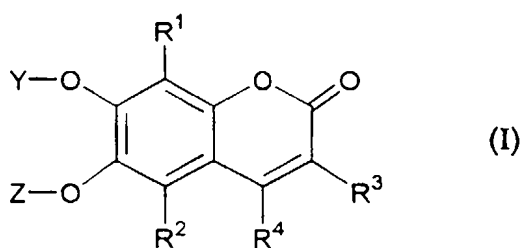
According to a second aspect of the invention there is provided a process in which a bacterial sample is cultured on a solid medium which comprises, in admixture, two chromogenic enzyme substrates, the enzymic reaction products of which are substantially non-diffusible in the solid medium, are capable of being detected optically in the presence of the respective substrates, and are different compounds, in which one of the substrates is an indoxyl compound and characterised in that the other of the substrates is an esculetin compound, preferably a 3,4-cyclohexenoesculetin compound.

Preferably one of the enzyme substrates is a substrate for a glycosidase, for instance β -galactosidase, α -galactosidase or β -glucosidase. Preferably one of the substrates is a substrate for a different glycosidase enzyme, although may alternatively be an esterase substrate. Preferably both substrates are for different glycosidase enzymes.

Most preferably one substrate is for α -galactosidase and the other is a substrate for β -galactosidase.

The esculetin substrate is substituted at the 6- or, preferably, 7-hydroxyl by a glycoside. Substituted 3,4-cyclohexenoesculetin compounds which produce non-diffusible complexes with metal ions, for instance, ferric ions, may also be used. Thus the cyclohexene ring may be substituted, or the coumarin ring system may be substituted, by one or more substituents.

The esculetin compound suitably has the general formula



wherein

each of R^1 and R^2 independently represents a hydrogen or a halogen atom or another group which does not interfere with subsequent iron chelation;

each of R^3 and R^4 independently represents a hydrogen atom or a (C_1-C_8) alkyl or $(C_6$ or $C_{10})$ aryl (C_1-C_8) alkyl or an optionally modified carboxyl-bearing group of the general formula $-CH_2(CH_2)_nCOX$, where n is a number from 0 to 3 and X represents a hydroxyl group or another hydrophilic group,

and, R^3 may alternatively represent an acyl group of the general formula $-COR$, in which R represent a (C_1-C_8) alkyl, $(C_6$ or $C_{10})$ aryl (C_1-C_8) alkyl or (C_5-C_8) cycloalkyl group,

provided that R^3 and R^4 between them contain at least three carbon atoms;

or R^3 and R^4 together with the carbon atoms to which they are attached form a (C_5-C_8) cycloalkene ring; and

one of Y and Z represents the enzymatically cleavable group and the other of Y and Z represents a hydrogen atom;

or a suitable salt or hydrate thereof.

Hereafter in this specification the term "compound" includes "salt" or "hydrate" unless the context requires otherwise.

As used herein the term "halogen" or its abbreviation "halo" means fluoro, chloro, bromo and iodo.

The expression "atom or group which does not interfere with iron chelation" refers to the fact that one of the principle means of detection of aglycones of general formula I is by chelation by means of hydroxyl groups at the 6 and 7 positions of the coumarin ring system. Groups which do not interfere with this chelation may be substituted at R¹ and/or R². Examples include hydrogen, hydroxyl, halogen or (C₁-C₆)alkyl. The halogen atom may be a fluorine atom or a chlorine atom and the lower alkyl group may be methyl, ethyl, propyl, butyl or benzyl.

As used herein the term "(C₁-C₈)alkyl" refers to straight chain or branched chain hydrocarbon groups having from one to eight carbon atoms. Illustrative of such alkyl groups are methyl, ethyl, propyl, isopropyl, butyl, isobutyl, *sec*-butyl, *tert*-butyl, pentyl, neopentyl, hexyl, heptyl and octyl. From one to four carbon atoms may be preferred.

As used herein the term "(C₁-C₁₀)alkyl" refers to straight chain or branched chain hydrocarbon groups having from one to ten carbon atoms. Illustrative of such alkyl groups are methyl, ethyl, propyl, isopropyl, butyl, isobutyl, *sec*-butyl, *tert*-butyl, pentyl, neopentyl, hexyl, heptyl, octyl, nonyl and decyl. From one to six carbon atoms may be preferred.

The term "(C₆ or C₁₀)aryl" includes phenyl and naphthyl.

As used herein, the term "(C₅-C₈) cycloalkene ring" refers to an alicyclic ring having from 5 to 8 atoms and having in addition one or more double bonds. Illustrative of such cycloalkenyl groups are cyclopentenyl, cyclohexenyl, cycloheptenyl and cyclooctenyl.

In compounds of this invention, the presence of an asymmetric carbon atom gives rise to enantiomers. The presence of several asymmetric carbon atoms give rise to diastereoisomers, each of which consists of two enantiomers, with the appropriate R or S stereochemistry at each chiral centre. The invention is understood to include all such diastereoisomers, optically active enantiomers and mixtures thereof.

The term "suitable salt" refers to a salt prepared by contacting a compound of formula I with an acid or base whose counterpart ion does not interfere with the intended use of the compound. Examples include the sodium salt or magnesium salt of a phosphate derivative or the salt formed from a primary, secondary or tertiary amine

where the compound or general formula I is a carboxylic acid. An example of a primary amine salt can be the cyclohexylammonium salt, a suitable secondary amine salt may be the piperidine salt and a tertiary amine salt may be the triethylamine salt.

Preferred compounds of general formula I include those in which, independently
5 or in any compatible combination:

R^1 is chlorine or, preferably hydrogen;

R^2 is chlorine or, preferably hydrogen;

R^3 is (C_1-C_4) alkyl, particularly butyl, or benzyl;

R^4 is (C_1-C_4) alkyl; or, $-CH_2(CH_2)_nCOX$, where n is a number from 0 to 3 and X
10 represents a hydroxyl group or one of the following hydrophilic groups, namely:

$-NHCH_2CONHCH_2CO_2H$

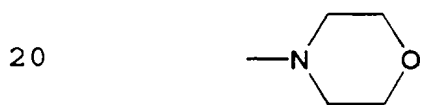
$-NHCH_2CONHCH_2CONHCH_2CO_2H$

$-NHCHCH_2CONH_2$

15 $-NHCHCH_2CONH_2$
 |
 CO_2H

$-NHCH_2CH_2SO_3H$

$-N(CH_2CO_2H)_2$, or,



R^3 and R^4 together with the carbon atoms to which they are attached form a (C_5-C_6) cycloalkene ring, preferably a cyclopentenyl or cyclohexenyl ring;

25 where R^3 is $-CH_2(CH_2)_nCOX$, where n is a number from 0 to 3, then the group X is as previously defined,

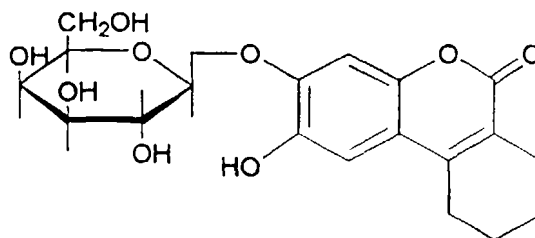
the enzymatically cleavable group represented by Y or Z is an α -or, preferably, β -linked sugar residue such as β -D-glucose, β -D-galactose, β -D-xylose, β -D-glycuronic acid or N-acetyl- β -D-glucosamine. Sugar residues derived from galactose especially β -
30 D-galaclopy-anosides are the most preferred compounds.

Compounds in which R^3 and R^4 together with the carbon atoms to which they are attached form a cyclopentene or a cyclohexene ring are especially preferred.

A preferred compound of general formula (I) is:

3,4-cyclohexenoesculetin- β -D-galactoside,

5



10

The enzymic reaction product of a 3,4-cyclohexenoesculetin substrate produces a brown black complex in the presence of ferric ion. The enzymic reaction product of a 5-bromo-4-chloro-3-indolyl compound produces a green or blue colour in the presence of oxygen. Other indoxyl derivatives are available, which have different substituents so as to generate a different coloured reaction product, for instance which is magenta, rose, blue, salmon red, and any of these can be used in place of the 5-bromo-4-chloro-3-indolyl compound. A bacterial colony which is positive for the enzyme which cleaves the esculetin substrate generates a black colour in the presence of ferric ions. Colonies which are positive for the enzyme which cleaves the 5-bromo-4-chloro-3-indolyl substrate produce a green colour. Colonies which are positive for both enzymes can be distinguished from colonies which are positive for the enzyme of which the indoxyl compound is a substrate but which are negative for the other enzyme or negative for both. The reaction product of the esculetin substrate masks the reaction product of the indoxyl substrate, however, so that colonies which are positive for both enzymes cannot necessarily be distinguished from those which are positive only for the enzyme of which the esculetin compound is a substrate.

25

The esculetin substrate is generally present in a concentration of about 200 to 500 mg/l in the agar medium, more preferably about 300 mg/l. The indoxyl substrate is present in amounts of up to 300 mg/l, although it is generally unnecessary to use concentrations higher than 100 mg/l. The amount is usually at least 35 mg/l, for instance about 70 mg/l. This concentration of ferric ions is usually about 400 to 1000 mg/l (based on ferric ammonium citrate) for instance about 500 mg/l.

30

Preferably the method of the second aspect of the present invention is for identifying the presence of *Salmonella*. The culturing of the bacteria is therefore preferably carried out in the presence of an inhibitor of other enterobacteria and/or a promoter for β -galactosidase.

5 According to a further aspect of the invention there is provided a new composition for use in the culturing of bacteria which comprises in admixture a first chromogenic enzyme substrate for β -galactosidase and a second chromogenic enzyme substrate, the substrates being selected such that the enzymic reaction products of the two enzymes are different compounds and is characterised in that the second substrate
10 is a substrate for α -galactosidase.

The new composition of this aspect of the invention is suitable for use in the process of the first aspect of the invention. Preferably the composition contains other components suitable for carrying out the culturing step of the bacteria, and thus contains one or more nutrients for bacterial growth, and preferably a support substance, for
15 instance a gelling substance such as agar. Preferably the composition is in a dry, hydratable form whereby it can be hydrated to form a ready-to-use culture medium. The medium preferably contains the other components useful in the culture medium as described above in connection with the process.

20 According to a further aspect of the invention there is provided a new composition for use in the culturing of bacteria comprising in admixture a first chromogenic enzyme substrate which is an indoxyl compound and a second chromogenic enzyme substrate, and is characterised in that the second enzyme substrate is an esculetin, preferably a 3,4-cyclohexenoesculetin, compound.

25 In this aspect of the invention, the composition also preferably contains one or more nutrients for bacterial growth as well as a support substance, for instance a gelling substance such as agar. The composition is preferably in hydratable form and should contain further ferric salt, which generates the black compound in the presence of the enzymic reaction product of the esculetin compound. Ferric ammonium citrate is conveniently used although ferric gluconate or other salts could be used as alternative
30 sources of ferric ions.

The compositions may be based on selective basal media which inhibit normal microbial flora and allow selective growth of *Salmonella*. Known media of this type are

bismuth sulphite agar, Brilliant green agar, Hektoen enteric agar and *Salmonella/Shigella* agar.

Other preferred embodiments of this further aspect of the invention are described above in connection with the novel processes. As mentioned above, it is believed that
 5 this is the first time that α -galactosidase has been used as a marker for *Salmonella* in the detection of *Salmonella* in a mixed sample.

Accordingly in a further aspect of the invention there is provided a new use of a chromogenic α -D-galactoside enzyme substrate to detect the presence of *Salmonella* species in a mixed species sample. Preferably the detection is carried out by culturing the
 10 mixed sample on an agar medium. Chromogenic α -galactosidase substrates are commercially available. The enzyme substrate is preferably 5-bromo-4-chloro-3-indolyl- α -D-galactopyranoside.

The invention is described further in the following example:

Example

15 The following base culture medium is made up. It encourages the growth of *Salmonella* at the expense of other enterobacteria by incorporation of desoxycholate.

DCA Hynes Base (per litre)

	Beef extract	5.0g
20	Balanced peptone No. 1	5.0g
	Sodium citrate	8.5g
	Sodium desoxycholate	5.0g
	Agar No. 2	12.0g

Chromogenic Mix (per litre)

25	5-Bromo-4-chloro-3-indolyl- α -D-galactopyranoside	70 mg
	3,4-cyclohexenoesclatin- β -D-galactopyranoside	300 mg
	Ferric ammonium citrate	500 mg
	Isopropyl- β -D-thiogalactopyranoside	30 mg

All of the above ingredients are dissolved in 1 litre of distilled water and autoclaved at 116°C for 10 minutes. The agar is then poured in sterile plastic petri dishes and allowed to set.

5 Evaluation

Members of the *Enterobacteriaceae* of known identity were obtained in pure culture and inoculated onto the new selective medium. All plates were incubated at 37°C for 18 hours and examined for colour production. 1020 of these strains were known to be *Salmonella* and had been consecutively isolated from faeces samples at both the
10 Freeman Hospital (120 strains) and the Newcastle Regional Public Health Laboratory (900 strains). Of the 1020 *Salmonella* strains, 1016 (99.6%) produced a green colony characteristic of *Salmonella*. Of the remaining four strains there were three strains which did not produce α -galactosidase and remained colourless. These were two strains of *Salmonella saint-paul* and one strain of *Salmonella branderup*. The remaining strain
15 was a β -galactosidase producing *Salmonella arizonae* which consequently produced a black colony.

Of the 300 non-*Salmonella*, only one strain produced a green colony typical of *Salmonella*. This was a highly atypical strain of *Escherichia coli* which did not produce β -galactosidase. 39 other strains of *E.coli* produced a typical black colony.

20 From the above results it can be seen that the culture medium including the substrates for α -galactoside and β -galactoside is extremely sensitive (99.6%) but still highly specific (99.9%) for the detection of *Salmonella*. Furthermore the results are easy to read.

CLAIMS

1. A process in which the following steps are carried out:

1. a sample suspected of containing *Salmonella* bacteria is cultured in the presence of a nutrient,

5 2. the bacterial culture is contacted with each of two enzyme substrates,

3. the presence of the enzymic reaction products of each of the substrates is assessed after step 2 to determine whether or not growth of *Salmonella* species has taken place,

in which the first substrate is a substrate for an enzyme for which *Salmonella* is negative, the process being characterised in that the second substrate is a substrate for α -galactosidase, and in that both substrates are chromogenic.

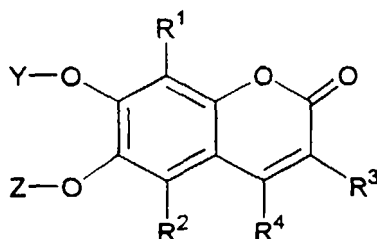
2. A process according to claim 1 in which one of the substrates is an indoxyl compound preferably a 5-bromo-4-chloro-3-indolyl compound.

3. A process according to claim 1 or claim 2 in which one of the enzyme
15 substrates is a esculetin compound, preferably a 3,4-cyclohexenoesculetin compound.

4. A process in which a bacterial sample is cultured on a solid medium which comprises, in admixture, two enzyme substrates, the enzymic reaction products of which are substantially non-diffusible in the solid medium, are capable of being detected optically in the presence of the respective substrates, and are different compounds, in
20 which one of the substrates is an indoxyl compound and characterised in that the other of the substrates is an esculetin compound.

5. A process acc. cl 4 in which the esculetin compound is a 3,4-cyclohexenoesculetin.

6. A process according to any of claims 3 to 5 in which the esculetin
25 compound has the general formula I



(I)

wherein

each of R^1 and R^2 independently represents a hydrogen or a halogen atom or
5 another group which does not interfere with subsequent iron chelation;

each of R^3 and R^4 independently represents a hydrogen atom or a (C_1-C_8) alkyl
or $(C_6$ or $C_{10})$ aryl (C_1-C_8) alkyl or an optionally modified carboxyl-bearing group of the
general formula $-CH_2(CH_2)_nCOX$, where n is a number from 0 to 3 and X represents a
hydroxyl group or another hydrophilic group, and, R^3 may alternatively represent an acyl
10 group of the general formula $-COR$, in which R represent a (C_1-C_8) alkyl, $(C_6$ or $C_{10})$ aryl
 (C_1-C_8) alkyl or (C_5-C_8) cycloalkyl group,

provided that R^3 and R^4 between them contain at least three carbon atoms;

or R^3 and R^4 together with the carbon atoms to which they are attached form a
 (C_5-C_8) cycloalkene ring; and

15 one of Y and Z represents the enzymatically cleavable group and the other of Y
and Z represents a hydrogen atom;

or a suitable salt or hydrate thereof.

7. A process according to claim 6 in which R^3 and R^4 , together with the
carbon atoms to which they are attached form a C_{5-8} cycloalkene ring, preferably a
20 cyclohexene ring.

8. A process according to any preceding claim in which one of the enzyme
substrates is 5-bromo-4-chloro-3-indolyl- α -D-galactopyranosidase.

9. A process according to any preceding claim in which one of the enzyme
substrates is 3,4-cyclohexenoescluletin- β -D-galactopyranoside.

25 10. A process according to any preceding claim in which the sample which
is cultured contains enterobacteria.

11. A process according to claim 11 in which the sample which is cultured is
derived from an enrichment process in which a direct sample is cultured in an enrichment
broth, for instance for 24 hours.

30 12. A process according to claim 10 in which the sample which is cultured in
the process is a direct non-enriched sample.

13. A process according to any of claims 10 to 12 in which the direct sample is of faeces.

14. A process according to any preceding claim in which the sample is cultured in the presence of an inhibitor of enterobacteria other than *Salmonella*,
5 preferably selected from brilliant green, bile salts, bismuth sulphite, Hektoen medium and desoxycholate salts.

15. A process according to any preceding claim in which the sample is cultured on a solid medium.

16. A process according to claim 15 in which the culture medium on which
10 culturing takes place contains both the enzyme substrates, the enzyme substrates being selected such that the enzymic reaction products of each of them is a different compound and is substantially non diffusible in the culture medium.

17. Use of a chromogenic α -galactosidase substrate in a process in which a mixed sample suspected of containing *Salmonella* species is cultured and in which the
15 enzymic reaction product of the substrate is detected optically, whereby a positive result is used as an indicator of the presence of *Salmonella* growth.

18. Use according to claim 17 in which the substrate is an indoxyl compound.

19. A composition for use in the culturing of bacteria which comprises in
20 admixture a first chromogenic enzyme substrate for β -galactosidase and a second chromogenic enzyme substrate, the substrates being selected such that the enzymic reaction products (the chromophors) of the two enzymes are different compounds and is characterised in that the second substrate is a substrate for α -galactosidase.

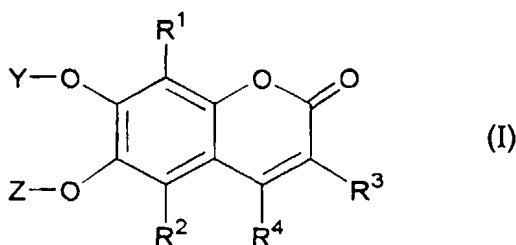
20. A composition according to claim 19 in which one of the substrates is an
25 indoxyl compound, preferably a 5-bromo-4-chloro-3-indolyl compound.

21. A composition according to claim 19 or claim 20 in which one of the substrates is an esculetin compound, preferably a 3,4-cyclohexenoesculetin compound.

22. A composition for use in the culturing of bacteria comprising in admixture a first enzyme substrate which is an indoxyl compound and a second enzyme substrate,
30 and is characterised in that the second enzyme substrate is a n esculetin compound.

23. A composition according to claim 21 or claim 22 in which the esculetin compound has the general formula I

17



5

wherein

each of R^1 and R^2 independently represents a hydrogen or a halogen atom or another group which does not interfere with subsequent iron chelation;

10 each of R^3 and R^4 independently represents a hydrogen atom or a (C_1-C_8) alkyl or $(C_6$ or $C_{10})$ aryl (C_1-C_8) alkyl or an optionally modified carboxyl-bearing group of the general formula $-CH_2(CH_2)_nCOX$, where n is a number from 0 to 3 and X represents a hydroxyl group or another hydrophilic group, and, R^3 may alternatively represent an acyl group of the general formula $-COR$, in which R represent a (C_1-C_8) alkyl, $(C_6$ or $C_{10})$ aryl
15 (C_1-C_8) alkyl or (C_5-C_8) cycloalkyl group,

provided that R^3 and R^4 between them contain at least three carbon atoms;

or R^3 and R^4 together with the carbon atoms to which they are attached form a (C_5-C_8) cycloalkene ring; and

one of Y and Z represents the enzymatically cleavable group and the other of Y
20 and Z represents a hydrogen atom;
or a suitable salt or hydrate thereof.

24. A composition according to claim 23 in which R^3 and R^4 , together with the carbon atoms to which they are attached form a C_{5-8} cycloalkene ring, preferably a cyclohexene ring.

25. A composition according to any of claims 18 to 23 which contains one or more nutrients for bacterial growth and a support substance, preferably a gelling substance, most preferably agar.

26. A composition according to any of claims 19 to 25 which is in a dry hydratable form.

30 27. A composition according to any of claims 19 to 26 which contains an inhibitor of enterobacterial growth other than of *Salmonella*, and is preferably selected

from brilliant green agar, agar containing bile salts, bismuth sulphite agar, agar containing desoxycholate salts, Hektoen enteric agar and *Salmonella/Shigella* agar.

28. A composition according to any of claims 19 to 27 which contains a promoter of β -galactosidase, preferably selected from lactose and isopropyl- β -D-thiogalactopyranoside.
- 5

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 98/01645

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12Q1/10 C12Q1/34 C12Q1/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KÉTYI: "Feeding by mucin and intestinal growth of some enteric bacterial pathogens" ACTA MICROBIOLOGICA HUNGARICA, vol. 35, no. 4, 1988, BUDAPEST, pages 389-395, XP002047084 cited in the application see page 394 --- -/--	1-5, 8-16, 18-22, 25-28

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

3 September 1998

Date of mailing of the international search report

11/09/1998

Name and mailing address of the ISA

European Patent Office, P.B. 5618 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Ceder, 0

INTERNATIONAL SEARCH REPORT

Int. onal Application No

PCT/GB 98/01645

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>WO 92 12259 A (BIO MERIEUX) 23 July 1992</p> <p>see abstract; examples see page 5, line 25 - page 6, line 15 & EP 0 516 817 A cited in the application</p>	<p>1,2,4,5, 9-12,14, 16, 18-20, 22,23, 27,28</p>
A	<p>JAMES ET AL: "Evaluation of cyclohexenoesucletin-beta-D-galactoside and 8-hydroxyquinoline-beta-D-galactoside as substrates for the detection of beta-galactosidase." APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 62, no. 10, October 1996, WASHINGTON, DC, pages 3868-3870, XP002047085 cited in the application see abstract</p>	<p>3,4,9, 21,22</p>
A	<p>CHEVALIER ET AL.: "X-alpha-Gal-based medium for simultaneous enumeration of bifidobacteria and lactic acid bacteria in milk" JOURNAL OF MICROBIOLOGICAL METHODS, vol. 13, no. 1, 1991, pages 75-83, XP002047086 see abstract</p>	<p>8,18</p>
A	<p>GB 2 050 418 A (API LABOR) 7 January 1981</p> <p>see abstract; examples see page 2, line 27</p>	<p>1-4,9, 10,15, 16,18, 21,22,25</p>
A	<p>US 5 210 022 A (ROTH JONATHAN N ET AL) 11 May 1993</p> <p>see abstract; table I see column 11, line 42 - line 52</p>	<p>1,2,4,9, 10,15, 16,25,28</p>
A	<p>WO 94 28163 A (FOSS ELECTRIC AS ;GLENSBJERG MARTIN (DK); HANSEN FLEMMING (DK)) 8 December 1994 see abstract</p>	<p>11</p>
A	<p>EP 0 614 896 A (KUREHA CHEMICAL IND CO LTD) 14 September 1994 see abstract</p>	<p>6,7,23, 24</p>

-/--

INTERNATIONAL SEARCH REPORT

Inter. Appl. No.

PCT/GB 98/01645

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>JAMES A L ET AL: "CYCLOHEXENOESCULETIN-BETA-D-GLUCOSIDE: A NEW SUBSTRATE FOR THE DETECTION OF BACTERIAL BETA-D-GLUCOSIDASE" JOURNAL OF APPLIED MICROBIOLOGY, vol. 82, no. 4, 1997, pages 532-536, XP002043328 cited in the application see the whole document</p> <p style="text-align: center;">-----</p>	<p>6,7,23, 24</p>

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte. onal Application No

PCT/GB 98/01645

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9212259	A	23-07-1992	FR 2671100 A	03-07-1992
			AT 135051 T	15-03-1996
			DE 69117744 D	11-04-1996
			DE 69117744 T	18-07-1996
			DK 516817 T	22-07-1996
			EP 0516817 A	09-12-1992
			ES 2084345 T	01-05-1996
			GR 3019798 T	31-07-1996
			PT 99945 A	29-01-1993
			US 5434056 A	18-07-1995

GB 2050418	A	07-01-1981	FR 2457323 A	19-12-1980
			US 4308348 A	29-12-1981

US 5210022	A	11-05-1993	US 5393662 A	28-02-1995
			US 5358854 A	25-10-1994

WO 9428163	A	08-12-1994	AT 157402 T	15-09-1997
			AU 6924694 A	20-12-1994
			DE 69405232 D	02-10-1997
			DE 69405232 T	02-01-1998
			EP 0701624 A	20-03-1996
			FI 955710 A	27-11-1995
			JP 9500522 T	21-01-1997
			NO 954899 A	01-02-1996

EP 0614896	A	14-09-1994	JP 6312925 A	08-11-1994
			AU 660870 B	06-07-1995
			AU 5643894 A	15-09-1994
			CA 2116617 A	03-09-1994
			CN 1101259 A	12-04-1995
			NO 940710 A	05-09-1994
			US 5455268 A	03-10-1995
